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The full extent to which hypoxia produces gene expression changes in human cells is unknown. We used late-generation oligonucleotide arrays to catalog hypoxia-induced changes in gene expression in HepG2 cells. Five paired sets of cultures were subjected to either control (room air-5% CO2) or hypoxic (1% O2-5% CO2) conditions for 24 h, and RNA was analyzed on an Affymetrix cDNA array containing 12,600 sequences. A statistically significant change in expression was shown by 2,908 sequences (1,255 increased and 1,653 decreased). The observed changes were highly concordant with published literature on hypoxic stress but showed relatively little overlap (12–22%) with changes in gene expression that have been reported to occur after heat stress in other systems. Of note, of these 2,908 sequences, only 387 (213 increased and 174 decreased) both exhibited changes in expression of twofold or greater and were highly expressed in at least three of the five experiments. We conclude that the effect of hypoxia on gene expression by HepG2 cells is broad, has a significant component of downregulation, and includes a relatively small number of genes whose response is truly independent of cell and stress type.

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Effect of hypoxia on gene expression by human hepatocytes (HepG2)

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cDNA array; hypoxic stress; cellular hypoxia; heat shock; cell stress response

CELLULAR HYPOXIA OCCURS in many common and clinically important conditions, ranging from environmental exposures (such as ascent to high altitude) to pathophysiological states (such as organ ischemia). Accordingly, human cells have evolved an ability to survive and adapt to reductions in ambient oxygen pressure. Functionally, these adaptations include compensatory changes that allow cells to survive the hypoxic exposure itself (for example, increases in anaerobic metabolism and initiation of a cell stress response) as well as responses that are designed to increase local oxygen delivery (such as production of angiogenic factors and erythropoietin) (30). In principle, a better understanding of these adaptive changes and the mechanisms that produce them may allow them to be selectively manipulated for therapeutic purposes (31, 39). For example, an ability to elicit a preconditioning hypoxic response in organs prior to surgery might in principle reduce the degree of ischemic injury that occurs as a result of clamping off the blood supply to that organ during the operation. Conversely, it has been proposed that the hypoxic microenvironment of solid tumors may trigger cellular adaptations that can be used to develop drugs and delivery systems that are specifically targeted to hypoxic cells (39). Accordingly, there is considerable scientific interest in identifying novel hypoxic responses.

Changes in gene expression are an integral part of the human cellular response to hypoxia (30, 31). To date, at least 50 mammalian genes have been identified which show a change in expression during hypoxic exposure, including a number of genes that are thought to be part of a nonspecific cellular response to stress. Furthermore, at least three important mechanisms for altering gene expression during hypoxia have been identified, namely, changes in transcription [mediated by well-described transcription factors including hypoxia-inducible factor 1 (HIF-1); Refs. 30, 31], stabilization of hypoxia-sensitive RNA species [such as vascular endothelial growth factor (VEGF)] (7), and the existence of internal ribosomal entry sites (IRES) that permit cap-independent translation of molecules such as VEGF even under severely hypoxic conditions (7).

The advent of DNA microarray technology has greatly expanded the ability of investigators to identify novel hypoxia-responsive genes (9, 15, 29) and has provided a tool that allows simultaneous examination of the effects of hypoxia on expression of multiple functionally related genes. For example, a recent study by Fink et al. (9) used both DNA array technology and real-time PCR to identify genes affected by hypoxia in a variety of human hepatocyte cell lines. In addition to identifying several previously unrecognized hypoxia-responsive genes, it was found that hypoxic exposure

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without reoxygenation led to an overall decrease in the number of transcripts expressed by cells, and interestingly, that hypoxic exposure in these human cell lines (without reoxygenation) did not lead to an increase in expression of heat shock proteins. Since publication of these experiments, DNA arrays have increased substantially in power and complexity, and it seemed likely that application of late-generation arrays would enable identification of even more hypoxia-responsive genes. We therefore decided to examine the effects of hypoxic exposure on mRNA expression in a human cell line, using Affymetrix GeneChip arrays containing ~12,600 sequences. A hepatocyte cell line was chosen because the liver is highly metabolically active and thus likely to be very sensitive to hypoxic exposures. To maximize the comparability of these experiments to the published literature, we chose a well-studied human cell line (HepG2 hepatocytes) and a hypoxic exposure (1% oxygen for 24 h) that is both conventional and known to produce somewhere between half-maximal and maximal activation of HIF-1 (14). Furthermore, as a first step toward assessing the extent to which the observed changes are part of a truly nonspecific cell stress response at the level of RNA expression, we have compared our results to published lists of mammalian genes that are known to be affected by heat shock.

MATERIALS AND METHODS

Cell culture. The hepatocellular carcinoma cell line HepG2 was purchased from the ATCC (catalog no. HB-8065; ATCC, Rockville, MD). Cells were grown in vented T-75 flasks containing 10 ml of a medium composed of minimum essential medium Eagle (ATCC) and 10% fetal bovine serum (GIBCO/Invitrogen, Carlsbad, CA), supplemented with penicillin-streptomycin (GIBCO/Invitrogen). Cells were grown to ~80% confluence in a room air-5% CO_2 incubator (range, 70–95%; mean ± SD, 81% ± 11%). In each paired experiment (control vs. hypoxic exposure), the media in each flask of cells to be used was replaced with fresh, prewarmed (37°C) media just before exposing the cells to 24 h of control (room air-5% CO_2) or hypoxic (1% O_2-5% CO_2) conditions as described below.

Test conditions. Cells were exposed to hypoxia by placing flasks of cultured cells into a custom-built airtight chamber designed to fit within a tissue culture incubator (5). It was superfused with a mixture of analyzed hypoxic gas consisting of 1.0–1.1% O_2 and 5.3% CO_2 (balance N_2) (Liberty Supply, Leominster, MA) that had first been bubbled through an inline water trap contained within the incubator to provide heat and humidity. The concentration of CO_2 used was chosen based on preliminary experiments, which revealed that a concentration of gas slightly greater than 5% was needed to compensate for line losses. The humidified gas fractions of oxygen and carbon dioxide within the chamber were measured by use of an inline oximeter/CO_2 analyzer (Alpha-Omega model 9500) connected to the outlet port of the chamber. At steady state, the gas concentration of CO_2 in the five experiments we report here was 5.2 ± 0.1% (mean ± SD) and the concentration of O_2 was 1.0 ± 0.05% (mean ± SD) (5). Cells exposed to control conditions were concurrently placed in a separate room air-5% CO_2 incubator. Both sets of cultures were left undisturbed throughout the 24-h exposure period. At the end of the 24-h exposure, cell survival under control and hypoxic conditions was assessed by the trypan blue exclusion method.

For the heat shock control experiments, cells grown under conditions identical to those used for hypoxia were placed in a 43°C water bath (inside a 5% CO_2 tissue culture incubator) for 30 min or maintained under control conditions (37°C, also in a 5% CO_2 incubator) and then returned to 37°C. RNA was isolated immediately upon return to 37°C (*time 0*) and at 1, 2, and 4 h after heat shock or control exposure.

RNA purification, RNA was extracted from cells after 24 h of control or hypoxic exposures with RNeasy kits (Qiagen, Valencia, CA), according to the directions of the manufacturer. At the conclusion of each hypoxic exposure, the culture flasks were removed from the incubator, and lysis of the cells was performed as quickly as possible to minimize the effects of reoxygenation. We estimate that the time required to do this was less than 2 min. The quality of the extracted RNA was assessed as recommended by Farrell (8), as described previously in detail (34). Samples were judged suitable for DNA array analysis only if the RNA was of a suitable yield (at least 25–35 μg), exhibited intact bands corresponding to the 18S and 28S subunits, and displayed no spurious peaks on a UV absorption spectrum in the range 230–320 nm. Furthermore, samples submitted for DNA array analysis were also analyzed by reverse transcription-PCR (RT-PCR) to ensure that a detectable hypoxic response had occurred at the level of mRNA expression, as judged by an increase in carbonic anhydrase IX (CA IX) under hypoxic conditions (Fig. 1), a gene which has been shown in several other tumor cell lines to be highly upregulated by hypoxia (40). Of the first eight experiments performed for purposes of oligonucleotide

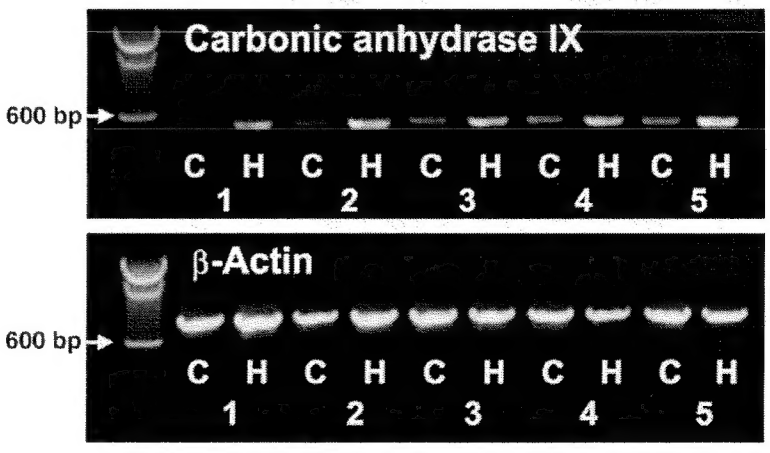

Fig. 1. RT-PCR showing the effect of hypoxia exposure on expression of carbonic anhydrase (CA) IX by HepG2 cells. C, control; H, hypoxia. Each paired set of samples is labeled with the experiment number (1 through 5). The arrows point to the 600-bp band in the ladder.

array analysis, five met our RNA quality criteria, and all of these showed an increase in CA IX expression as judged by RT-PCR (Fig. 1).

GeneChip array hybridization. Transcript profiling with Affymetrix GeneChips (Affymetrix, Santa Clara, CA) was performed using HG-U95Av2 GeneChips containing ~12,600 sequences (representing ~11,300 unique GenBank accession numbers), as previously described (34).

Data analysis. Data analysis was performed using Microsoft Excel, Microsoft Access, and SigmaStat 2.0 for Windows. The fold changes in gene expression reported by the Affymetrix software (MAS 4.0) in the paired experiments were used to determine whether a statistically significant change in expression had occurred, by computing geometric means and 95% confidence intervals as described in detail previously (34).

Where noted in this paper, sequences that showed a statistically significant change in expression were filtered by two post hoc criteria. First, sequences were excluded if they were labeled as "absent" by the GeneChip-reading software in more than half of the control samples (for downregulated genes) or in more than half of the hypoxic samples (for upregulated genes). Second, sequences were excluded if the change in geometric mean expression was less than twofold.

Confirmatory RT-PCR. Samples submitted for GeneChip array analysis were subjected to a separate poly-T primed RT-PCR using a commercially available kit (Retroscript First-Strand Synthesis Kit; Ambion, Austin, TX), following the manufacturer's instructions. Each resulting mixture (consisting of cDNA and unreacted primers) was diluted to 50 ng/μl, and 100-ng aliquots were subjected to 31 cycles of PCR at a Tm of 60°C, using primers designed to recognize CA IX, MAX-interacting protein-1 (MXI-1), dual-specificity phosphatase-1 (DUSP-1), zinc finger protein 36 (ZFP-36), outer mitochondrial membrane translocator 34 (TOM-34), interferon-inducible protein 30 (IFI-30), or cyclophilin A. The β-actin primers were obtained from a commercial source (Clontech, Palo Alto, CA) and the other primers were designed using PRIMER-3 software. The sequences (5′-3′) of these primers were as follows: CA IX tatctgcactcctgccctct (forward primer), gctggcttctcacattctcc (reverse primer), designed to yield a 475-bp amplicon; MXI-1, tctcccatggagaagtggac (forward), agaggatggcatctccaatg (reverse), designed to yield a 450-bp amplicon; DUSP-1, aagaatgctggaggaagggt (forward), ttcagcaaatgtcttgacgc (reverse), designed to yield a 543-bp amplicon; ZFP-36, gtcaccctctgccttctctg (forward), ggtcaggggagtgggttaat (reverse), designed to yield a 454-bp amplicon; TOM-34, acatggttgttgcaccagaa (forward), atggacactgaccaaggagg (reverse), designed to yield a 546-bp amplicon; IFI-30, tgcaaattcaacaaggtgga (forward), ggtaagtagcaggtgccgag (reverse), designed to yield a 483-bp amplicon; and cyclophilin A, aggtcccaaagacagcagaa (forward), tgtccacagtcagcaatggt (reverse), designed to yield a 406-bp amplicon. The samples subjected to heat shock were analyzed using the following published (34) HSP 70B′ primers (5′-3′): aggagatctcgtccatggtg (forward), ttccatgaagtggttcacga (reverse), designed to yield a 380-bp amplicon. The sequences of the HSP 70B′ primers are complementary to both HSP 70B′ (HSPA6) and to HSP 70B (HSPA7), a poorly expressed pseudogene of nearly identical sequence that encodes a truncated (and probably nonfunctional) protein (24).

RESULTS

Confirmation of a hypoxic response at the level of mRNA expression. RNA of satisfactory quality was obtained from five experiments in which paired flasks of cells were exposed to either control (room air-5% CO_2) or hypoxic (1% O_2-5% CO_2) conditions for 24 h. In all five of these experiments, internal oxygen concentration (Fio_2) in the hypoxia chamber had decreased to 1.5% or less within 30 min. At 60 min and 24 h, respectively, the mean values of Fio_2 in the hypoxia chamber (±SD) were 1.2 ± 0.08% and 1.0 ± 0.05%. Control cells were maintained in a standard room air-5% CO_2 incubator. There was no statistically significant difference in the survival of cells exposed to hypoxia vs. control conditions [cell survival of 96 ± 2% (±SE) under control conditions and 95 ± 2% under hypoxic conditions by trypan blue exclusion; $P = 0.29$ by paired t-test].

The expression of CA IX, a molecule known to be substantially upregulated by hypoxic stress in other tumor cell lines (40), was substantially increased in cells exposed to hypoxic conditions compared with those maintained under control conditions (Fig. 1). By contrast, there was no apparent effect on the expression of β-actin.

GeneChip array results. Under control conditions, HepG2 cells expressed a mean (±SE) of 5,493 ± 139 sequences as "present" or "marginal." Under hypoxic conditions, these cells expressed 5,182 ± 130 sequences. This decrease in the number of transcripts expressed showed a trend toward statistical significance ($P = 0.12$ by paired t-test, $n = 5$). The trend was also noted when the analysis was limited to only those sequences identified as "present" by the GeneChip-reading software. Under control conditions, 5,247 ± 137 sequences were identified as "present" compared with 4,930 ± 128 under hypoxic conditions ($P = 0.11$ by paired t-test, $n = 5$). Importantly, 5,393 sequences were expressed as "present" or "marginal" in at least 3 experiments under control conditions (3,944 in all 5), and 5,051 were expressed in at least 3 experiments under hypoxic conditions (3,677 in all 5).

Of the ~12,600 sequences on the GeneChip, 2,908 showed a statistically significant change in expression between the control and hypoxic condition (as defined by geometric mean 95% confidence intervals that excluded unity). Of these, 1,255 showed an increase in expression, and 1,653 showed a decrease in expression. However, only 2,090 of these sequences (905 increased and 1,185 decreased) were expressed (i.e., identified as "present" or "marginal") in at least 3 experiments under control conditions (for the decreased genes) or under hypoxic conditions (for the increased genes). Furthermore, of these 2,090 sequences, only 387 (213 increased and 174 decreased) showed a mean change in expression of twofold or greater. Based on UniGene number assignments, we estimate that these 387 sequences represent about 352 different genes (185 increased, 167 decreased).

Effect of hypoxia on control gene expression. Hypoxia had little or no effect on the expression of several control ("housekeeping") sequences (Table 1). Because glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is known to be increased by hypoxia and is a target for HIF-1 (31), it was not included as a control.

Table 1. *Effect of hypoxia on important control sequences*

Common Name(s)	GenBank No.	UniGene No.	Change in Expression, fold (geometric mean, 95% CI)
Cyclophilin A	X52851	Hs.342389	0.98 (0.93–1.0)
Ribosomal protein L37a	L06499	Hs.296290	1.2 (0.99–1.4)
Ribosomal protein L41	Z12962	Hs.356795	1.2 (1.0–1.5)
H3F3A; H3 histone, family 3A	M11353	Hs.367720	0.95 (0.80–1.1)
Alu-Sq family consensus sequence	U14573	NC	1.1 (0.94–1.3)
β-Actin			
5′ End	X00351	Hs.288061	1.5 (1.1–2.1)†
Middle sequence	X00351	Hs.288061	1.4 (1.1–1.9)†
3′ End	X00351	Hs.288061	1.3 (1.0–1.7)†

Except where noted, UniGene numbers in this manuscript are from Build 157. †Statistically significant change in expression. NC, no corresponding Unigene number.

We found no significant changes in expression of cyclophilin A, the Alu-Sq family consensus sequence, ribosomal proteins L37a and L41, or the replication-independent histone H3FA3. We did observe a small but statistically significant change in β-actin expression; however, the magnitude of this change in expression (1.3- to 1.5-fold) was smaller than what is typically detectable by RT-PCR and was not readily apparent in our RT-PCR experiments (Fig. 1)

In addition to the common housekeeping sequences listed in Table 1, we also examined the effect of hypoxia on the sequences most highly expressed by cells under control conditions. Of the 10 sequences most highly expressed under control conditions, only 2 (both corresponding to GAPDH) showed a statistically significant change in expression as a result of hypoxia. The 8 unaffected sequences were ferritin light polypeptide, eukaryotic translation elongation factor 1 α1 (2 sequences), the Alu-sq family consensus sequence, ribosomal proteins L37a and L41, albumin, and laminin receptor 1.

Effect of hypoxia on known hypoxia-sensitive genes. To verify that our experimental methods appropriately detected changes in expression that would be expected from a conventional hypoxic exposure, we examined the changes in expression that occurred in a list of genes that are known targets for HIF-1, as reported in two recent reviews by Semenza (30, 31) (Table 2). To this list, we added a number of other genes that have specifically been shown in HepG2 cells to be increased by hypoxia (3, 9, 10, 12, 19, 38). We did not apply post hoc filtering to the data in Table 2, as post hoc filtering eliminates genes from consideration based on criteria other than statistical significance. Our results were highly consistent with these previous findings. We identified a probe on the U95Av2 array for 36 of 38 target genes listed, and of these, 29 (81%) showed a statistically significant increase in expression as a result of hypoxic exposure. Only one (haptoglobin) showed a significant decrease in expression. Of the remaining sequences, three (heme oxygenase 1, α-1 acid glycoprotein 1, and p21) were expressed in at least three of the five experiments but did not show a statistically significant change in expression as a result of hypoxia. None of the three other sequences (α1B-adrenergic receptor, nitric oxide synthase 2 (iNOS), and VEGF receptor FLT-1) were expressed in a majority of our experiments. Of these, the α1B-adrenergic receptor is known not to be expressed by HepG2 cells (2), and expression of nitric oxide synthase 2 (iNOS) by HepG2 cells is both very low under basal conditions (1) and shows a blunted increase in expression under interferon-stimulated conditions (1, 36).

Additionally, two genes (erythropoietin and IGFBP-2) showed a statistically significant change in expression but were not detected as "present" or "marginal" in at least three of our five experiments, suggesting very low (but inducible) levels of expression of these transcripts in this cell line under our culture conditions.

Our results were also highly congruent with the effects of 1% oxygen exposure on HepG2 gene expression as reported on Northern analysis by Wenger et al. (38). We identified sequences on the GeneChip corresponding to 9 of 10 genes they reported to be upregulated by hypoxia, and of these, 7 (78%) showed a statistically significant increase in expression in our experiment (VEGF, erythropoeitin, aldolase A, transferrin, α-1 antitrypsin, α-1 antichymotrypsin, and β-actin, Tables 1 and 2). α-1-Acid glycoprotein, which was marginally elevated in their study, was not significantly affected in ours (Table 2). Haptoglobin, by contrast, was significantly downregulated in our experiment (Table 2). Among genes reported to be unaffected by hypoxia by Wenger et al. (38), α-fibrinogen and hemopexin were also unaffected in our study, and β-fibrinogen showed only a small though significant decrease in expression (0.78-fold, 95% CI, 0.69–0.89).

We also compared our results to a list of 20 transcripts that were recently reported to be downregulated by hypoxia in HepG2 cells by real-time PCR methods (9). We were able to identify sequences on the GeneChip arrays corresponding to 17 of these 20 transcripts. Of these, five (HSC 70, HSP 70/90 organizing protein/transformation-sensitive protein-1, FLAP endonuclease 1, CDC25B, and chromatin assembly factor 1 p48 subunit) showed a statistically significant decrease in expression, consistent with previously reported results. Two others (ICAM-1 and ephrin receptor EphA2) showed a statistically significant increase in expression, in contradiction to previously reported results. The remaining 10 showed changes in expression that were not statistically significant (6 decreases and 4 increases).

Other genes affected by hypoxia. Although 2,908 transcripts showed a statistically significant change in expression as a result of hypoxic exposure, only 387 also met our post hoc filter criteria. These post hoc filter criteria were intended to maximize the likelihood that the cDNA array findings will be reproducible by techniques such as RT-PCR, by requiring the presence of both a strong signal under at least one condition

Table 2. *Effect of hypoxia on known hypoxia-responsive transcripts*

Common Name(s)	GenBank or TIGR No.	UniGene No.	Change in Expression, fold (geometric mean, 95% CI)
Genes widely known to be upregulated by hypoxia via HIF-1 (30, 31)			
Adenylate kinase 3	X60673	Hs.274691	1.5 (1.2–1.9)
α1B-Adrenergic receptor	U03865	Hs.123055	1.4 (0.71–2.9)*†
Adrenomedullin	D14874	Hs.394	15 (6.2–35)
Aldolase A	X05236	Hs.273415	1.8 (1.2–2.8)
Aldolase C	AF054987	Hs.155247	2.0 (1.7–2.4)
Endothelin 1	Y00749	Hs.2271	N/A
Enolase 1 (alpha)	M55914	Hs.254105	1.7 (1.3–2.4)
Erythropoietin	X02158	Hs.2303	7.6 (2.9–20)†
Glucose transporter 1	K03195	Hs.169902	1.4 (1.2–1.6)
Glucose transporter 3	M20681	Hs.7594	7.0 (3.6–14)
Glyceraldehyde phosphate dehydrogenase	M33197	Hs.169476	
3′ End			1.5 (1.2–2.0)
5′ End			1.8 (1.4–2.3)
Middle			1.7 (1.2–2.3)
Heme oxygenase 1	Z82244	Hs.202833	1.2 (0.40–3.6)*
Hexokinase 1	X66957	Hs.118625	N/A
Hexokinase 2	Z46376	Hs.198427	4.8 (3.4–7.0)
Insulin-like growth factor 2 (somatomedin A)	HT3739	Hs.349109	1.6 (1.3–2.0)
Insulin-like growth factor binding protein 1 (IGFBP-1)	M74587	Hs.102122	4.1 (1.1–15)
Insulin-like growth factor binding protein 2 (IGFBP-2)	X16302	Hs.162	2.0 (1.2–3.4)†
Lactate dehydrogenase A	X02152	Hs.2795	1.8 (1.1–2.8)
Nitric oxide synthase 2 (iNOS)	U31511	Hs.193788	0.67 (0.29–1.6)*†
p21 (WAF-1, Cip1)	U03106	Hs.179665	0.99 (0.38–2.6)*
p35srj (CITED-2, mrg-1)	U65093	Hs.82071	4.5 (2.4–8.4)
Phosphofructokinase L	X15573	Hs.155455	1.5 (1.3–1.7)
Phosphoglycerate kinase 1 (PGK-1)	V00572	Hs.78771	2.4 (1.7–3.4)
Pyruvate kinase M	M26252	Hs.198281	2.0 (1.4–2.8)
Transferrin	S95936	Hs.356450	1.3 (1.1–1.5)
Transferrin receptor	X01060	Hs.77356	1.4 (1.1–1.6)
Vascular endothelial growth factor (VEGF)	M63978	Hs.73793	13 (7.1–25)
	AF022375	Hs.73793	4.0 (3.2–4.9)
	AF024710	Hs.73793	3.7 (2.8–4.7)
VEGF receptor FLT-1	U01134	Hs.381093	1.1 (0.65–1.9)*†
	S77812	Hs.381093	0.86 (0.40–1.8)*†
Other genes previously reported to be upregulated by hypoxia in HepG2 cells			
Plasminogen activator inhibitor-1 (PAI-1) (9,10)	J03764	Hs.82085	73 (7.8–670)
	M14083	Hs.82085	13 (7.7–22)
Platelet derived growth factor beta (12)	M12783	Hs.1976	22 (14–36)
Insulin-like growth factor binding protein 3 (IGFBP-3) (9)	M35878	Hs.77326	18 (6.2–51)
	M35878	Hs.77326	3.1 (1.3–7.1)
Dual-specificity phosphatase-1 (DUSP-1, MAP kinase phosphatase-1, MKP-1) (32)	X68277	Hs.171695	23 (9.2–58)
c-fos (3)	V01512	Hs.25647	10 (3.8–28)
	V01512	Hs.25647	3.5 (2.1–5.7)
	K00650	Hs.25647	12 (2.4–55)†
c-jun (3, 19)	J04111	Hs.78465	5.4 (2.9–9.9)
	J04111	Hs.78465	2.6 (1.8–3.6)
α1-Antitrypsin; SERPIN A1 (38)	X01683	Hs.297681	1.3 (1.1–1.4)
α1-Antichymotrypsin; SERPIN A3 (38)	X68733	Hs.234726	1.6 (1.3–2.0)
Haptoglobin (38)	X89214	Hs.75990	0.78 (0.63–0.57)
	X00442	Hs.75990	0.45 (0.22–0.95)
α1-Acid glycoprotein 1; orsomucoid 1; orm-1 (38)	X02544	Hs.572	1.0 (0.80–1.3)*

*Change in expression is not statistically significant. †Expression was very low (or absent) in these cells under both control and hypoxic conditions. NA, no probe corresponding to this sequence was found in the array.

(control vs. hypoxia) in a majority of experiments and a large fold change in expression between the two conditions (at least 2-fold) on average over all five experiments. Of the 387 sequences that met our post hoc filter criteria, 59 were of unknown or unclear function. Of the remaining 328, about ¾ belonged to 1 of 9 functional categories: metabolic/biosynthetic enzymes and metabolic regulatory proteins (61 sequences); cell growth, proliferation, and differentiation (52 sequences, including at least 5 transcripts known to encode proteins that affect the myc system); transcription (30 sequences); signal transduction (28 sequences, including at least 4 transcripts known to encode proteins that affect MAP kinase pathways); immune function (19 sequences); growth factors and related proteins (19 sequences); RNA processing and conformation (20 sequences); membrane transport (15 sequences); and cytoskeleton and cell structure related proteins (10 sequences).

Table 3. *Other transcripts significantly increased by hypoxia*

Functional Class	Common Name(s)	GenBank or TIGR No.	UniGene No.	Change in Expression, fold (geometric mean, 95% CI)	Examples of Other Systems in Which Expression of This Transcript is Similarly Affected by Hypoxia	Reference(s)
Carbonic anhydrase	Carbonic anhydrase IX	X66839	Hs.63287	78 (27–222)	Bone, breast, cervical, alveolar pneumocytes A549	40
Apoptosis	STK17A; serine/threonine kinase 17a (apoptosis-inducing)	AI961743	Hs.9075	7.6 (4.4–13)		
	GADD45B; growth arrest and DNA-damage-inducible, beta	AF078077	Hs.110571	5.9 (2.0–18)	Head and neck squamous cell carcinoma (GADD45, subtype unspecified)	15
	BCL2/adenovirus E1B 19kDa interacting protein 3-like; NIX	AF079221	Hs.132955	5.3 (3.3–8.5)	Breast and bladder carcinomas	35
	Nuclear hormone receptor TR3; NP10; NR4A1; NUR77	L13740	Hs.1119	4.1 (1.8–9.4)	Rat brain	37
Cell growth, proliferation, and differentiation	BHLHB2; Basic helix-loop-helix domain containing, class B, 2; DEC-1	AB004066	Hs.171825	7.4 (3.2–17)	Pancreatic, bladder and breast carcinomas; alveolar pneumocytes A549	41, 42
	Cysteine-rich angiogenic inducer 61; IGFBP-10	Y11307	Hs.8867	5.6 (2.9–11)		
	UNC93A; Unc93 homolog A	AL021331	Hs.267749	5.1 (3.9–6.7)		
	FHL2; four and a half LIM domains 2	U29332	Hs.8302	5.0 (2.2–11)		
	MADH3; MAD (mothers against decapentaplegic, *Drosophila*) homolog 3; SMAD3; HMAD-3	U68019	Hs.288261	4.5 (1.9–11)		
Cell adhesion	ITGA2; integrin, alpha 2; CD49B	X17033	Hs.271986	4.1 (1.9–8.9)		
Endocytosis	NPTXR; neuronal pentraxin receptor	AL008583	Hs.91622	4.7 (3.0–7.4)		
Extracellular protein	Trefoil factor-1; TFF1	AA314825	Hs.350470	9.3 (2.5–34)	Caco-2 colonic epithelial cells	11
Growth factors and related proteins	Inhibin-α	M13981	Hs.1734	17 (7.4–38)	Rat brain	17
	FSTL3; follistatin-like 3 (secreted glycoprotein)	U76702	Hs.25348	8.0 (2.0–33)		
	AXL; AXL receptor tyrosine kinase	M76125	Hs.83341	5.8 (4.0–8.4)		
	INHBB; inhibin, beta B (activin AB beta polypeptide)	M31682	Hs.1735	4.3 (1.9–9.8)		
Immune function	MIP-3-alpha	U64197	Hs.75498	4.7 (1.9–12)		
	Herpesvirus entry mediator; HVEM; TR2; LIGHTR	U70321	Hs.279899	4.1 (1.9–9.1)		
Membrane protein	Genethonin 1	AF062534	Hs.109590	6.9 (3.3–14)		
Membrane transport	SLC6A8; Solute carrier family 6 (neurotransmitter transporter, creatine), member 8; creatine transporter	U17986	Hs.187958	13 (3.2–54)		
Metabolism and biosynthesis	PFKFB4; 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 4	D49818	Hs.198278	11 (2.1–57)		
	PDK-1; pyruvate dehydrogenase kinase 1	L42450	Hs.339787	6.9 (2.9–17)		
	Adipose differentiation related protein; ADRP	X97324	Hs.3416	6.3 (4.6–8.6)		
	GBE1; glycogen branching enzyme 1	L07956	Hs.1691	5.3 (3.9–7.3)		
	PFKFB3; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	D49817	Hs.195471	4.6 (3.3–6.4)	Hepatocytes Hep3B; retinal pigment epithelial cells	18
	ENO2; Enolase 2, (gamma, neuronal)	X51956	Hs.146580	4.6 (2.6–7.9)		
	Phosphoglycerate kinase splice variant; PGK-1 mutant	S81916	Hs.380429	4.5 (2.6–7.7)		

Continued

Table 3.—*Continued*

Functional Class	Common Name(s)	GenBank or TIGR No.	UniGene No.	Change in Expression, fold (geometric mean, 95% CI)	Examples of Other Systems in Which Expression of This Transcript is Similarly Affected by Hypoxia	Reference(s)
Myc system	MAX-interacting protein 1; MXI-1	L07648	Hs.118630	8.8 (5.5–14)		
	N-myc downstream regulated; NDRG-1; DRG-1; differentiation-regulated gene 1; protein regulated by oxygen 1 (PROXY-1)	D87953	Hs.75789	8.1 (5.0–13)	Trophoblasts; breast carcinoma; alveolar pneumocytes A549	23, 28
Posttranslational processing	LOX; lysyl oxidase	L16895	Hs.102267	5.9 (2.6–13)		
	B cell RAG associated protein; *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase	AB011170	Hs.6079	6.7 (3.7–12)		
Protein degradation	Neuroserpin; HGNC	Z81326	Hs.78589	8.1 (4.8–14)		
RNA editing	APOBEC-3C; APOBEC1-like	AA806768	Hs.8583	11 (3.0–41)		
RNA stability	Zinc finger protein Zfp-36; tristetraprolin; GOS24	M92843	Hs.343586	13 (6.9–23)		
Signal transduction	SHB adaptor protein	X75342	Hs.379206	7.5 (2.0–28)		
	Protein tyrosine phosphatase, receptor type, M	X58288	Hs.154151	6.0 (2.3–16)		
	PPP1R3C; protein phosphatase 1, regulatory (inhibitor) subunit 3C; PPP1R5	Y18207	Hs.303090	4.9 (2.4–10)		
	Protein tyrosine phosphatase, receptor type, M	X58288	Hs.154151	4.7 (1.9–12)		
Transcription	HIVEP2; human immunodeficiency virus type I enhancer binding protein 2	AL023584	Hs.75063	4.9 (3.0–8.0)		
	JUNB; Jun B proto-oncogene	X51345	Hs.198951	4.6 (1.3–16)	Rat PC-12 pheochromocytoma	27
	PLU-1; putative DNA/chromatin binding motif	AJ132440	Hs.143323	4.3 (1.5–12)		
	MAFF; mafF; V-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein F; v-maf	AL021977	Hs.51305	4.0 (2.4–6.8)		
Unknown	KIAA 1199	AL049389	Hs.50081	5.3 (3.7–7.8)		
	KIAA1053	AB028976	Hs.173571	4.6 (3.3–6.3)		

We have included all sequences not tabulated elsewhere in this report that were increased by at least 4-fold and that met our post hoc filter criteria.

Additional sequences that were significantly and strongly affected by hypoxic exposure in our experiment are listed in Tables 3 and 4. Where possible, we have also listed examples of other systems in which the mRNA transcript in question is similarly affected by hypoxia. Many of the genes that were most strongly (≥4-fold) upregulated by hypoxia in this experiment (Table 3) have been reported to be similarly affected by hypoxia in other systems. By contrast, we found literature precedent for very few of the genes downregulated by hypoxia in this experiment (as noted above and as illustrated in Table 4).

Confirmatory experiments. We performed confirmatory RT-PCR on three highly upregulated and two highly downregulated genes. To our knowledge, only one of these (dual specificity phosphatases, DUSP-1) has been demonstrated to be affected by hypoxia both in HepG2 cells (32) and in other systems (16). In each case the RT-PCR results confirmed the cDNA array findings (Fig. 2). Hypoxia induced a strong increase in expression of MXI-1, DUSP-1, and ZFP-36. Hypoxia decreased expression of IFI-30 and TOM-34. Also, consistent with our cDNA array expression data, there was no detectable effect of hypoxia on the expression of cyclophilin A.

Effect of hypoxia on expression of heat shock proteins and chaperonins. Heat shock proteins are known to be increased by a wide variety of cellular stressors in non-human systems. However, a recent report found a downregulatory effect of hypoxia on heme oxygenase-1 (HSP-32) in human umbilical vein endothelial cells, coronary artery endothelial cells, and astrocytes (21). Additionally, hypoxia has been reported to decrease expression of HSP-70 in human microvascular endothelial cells (22), and a recent experiment using cDNA arrays and real-time PCR found a downregulatory effect of hypoxia on a limited number of HSPs in human hepatocytes cell lines (including HepG2) (9). To further clarify the effect of hypoxia on HSP and chaperonin expression in this human cell line, we examined the effect of hypoxia on the expression of 69 sequences including cyclophilin A, at least one representative of every major family of HSP known in humans, ubiquitins B and C, and every HSP probe sequence on the U95A array previously identified as being affected by heat shock in human peripheral blood mononuclear

Table 4. *Examples of transcripts significantly decreased by hypoxia*

Functional Class	Common Name(s)	GenBank or TIGR No.	UniGene No.	Change in Expression, fold (geometric mean, 95% CI)
Cell adhesion	DiGeorge syndrome critical region gene 2	D79985	Hs.2491	0.11 (0.026–0.45)
Cell growth, proliferation, and differentiation	POLE2; polymerase (DNA directed), epsilon 2	AF025840	Hs.99185	0.33 (0.20–0.55)
	Activator of S phase kinase; ASK; Dbf4	AB028069	Hs.152759	0.32 (0.24–0.42)
	DTYMK; deoxythymidylate kinase; thymidylate kinase; CDC8	L16991	Hs.79006	0.21 (0.061–0.73)
	Galectin-2	AL022315	Hs.113987	0.19 (0.059–0.58)
	TFDP1; transcription factor Dp-1	L23959	Hs.79353	0.18 (0.092–0.36)
	CDC6; cell division cycle 6	U77949	Hs.69563	0.18 (0.092–0.33)
	DOC-1R; tumor suppressor deleted in oral cancer-related 1	AF089814	Hs.379039	0.17 (0.064–0.44)
	PITX1; paired-like homeodomain transcription factor 1	U70370	Hs.84136	0.16 (0.080–0.33)
Coagulation	GGCX; gamma-glutamyl carboxylase; vitamin K-dependent carboxylase	L17128	Hs.77719	0.31 (0.25–0.39)
	Antithrombin III	L00190	Hs.75599	0.28 (0.090–0.88)
Cytoskeleton and cell structure	MYO10; myosin X	AB018342	Hs.61638	0.26 (0.097–0.71)
Growth factors and related proteins	FUS1; lung cancer candidate; PDGF-associated protein; PAP	AF055479	Hs.8186	0.21 (0.10–0.42)
	FGFR1; fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	X66945	Hs.748	0.11 (0.027–0.42)
Immune function	FAP48; FKBP-associated protein	U73704	Hs.49105	0.32 (0.14–0.75)
	Gamma-interferon inducible protein 30; IFI-30; lysosomal thiol reductase, interferon-inducible; GILT	J03909	Hs.14623	0.063 (0.021–0.19)
MAP kinase pathway	Ras inhibitor INF	HT511	Hs.324178	0.26 (0.12–0.55)
	MAP3K11; mitogen-activated protein kinase kinase kinase 11	L32976	Hs.89449	0.26 (0.10–0.66)
Membrane transport	CTNS; cystinosis, nephropathic; cystinosin	AJ222967	Hs.64837	0.28 (0.16–0.48)
	TOM34; translocator of the outer mitochondrial membrane 34	U58970	Hs.76927	0.15 (0.032–0.67)
Metabolism and biosynthesis	PCYT2; phosphate cytidylyltransferase 2, ethanolamine	D84307	Hs.226377	0.33 (0.21–0.52)
	GYG2; glycogenin 2	U94362	Hs.380757	0.31 (0.16–0.61)
	ARSDR1; androgen-regulated short-chain dehydrogenase/reductase 1	AA126515	Hs.179817	0.28 (0.084–0.91)
	NQO2; NAD(P)H dehydrogenase, quinone 2	U07736	Hs.73956	0.26 (0.090–0.73)
	GART; phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	X54199	Hs.82285	0.26 (0.084–0.77)
	CES2; carboxylesterase 2 (intestine, liver)	Y09616	Hs.282975	0.25 (0.11–0.56)
	CTPS; CTP synthase	X52142	Hs.251871	0.19 (0.15–0.24)
	GPI-H; PIGH; phosphatidylinositol glycan, class H	L19783	Hs.177	0.18 (0.11–0.31)
	HMBS; hydroxymethylbilane synthase	M95623	Hs.82609	0.17 (0.046–0.64)
	ALDH1B1; aldehyde dehydrogenase 1 family, member B1; aldehyde dehydrogenase 5	M63967	Hs.169517	0.16 (0.077–0.35)
Metabolic enzyme transcription	Bile acid receptor; farnesoid X-activated receptor; FXR	U68233	Hs.171683	0.16 (0.084–0.32)
Posttranslational processing	SPS; selenophosphate synthetase; selenium donor protein	U34044	Hs.124027	0.26 (0.11–0.59)
	Ubiquitin-like 3; UBL-3	AL080177	Hs.173091	0.20 (0.067–0.61)
RNA processing	U5-100K; Prp28; U5 snRNP 100-kDa protein	AF026402	Hs.168103	0.19 (0.13–0.27)
RNA stability	TARBP2; TAR (HIV) RNA binding protein 2	U08998	Hs.326	0.16 (0.060–0.44)
Signal transduction	RAC3; Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	AI570572	Hs.45002	0.15 (0.027–0.85)
	PSK-H1; protein serine kinase H1	AI767675	Hs.150601	0.12 (0.097–0.14)
	KIAA 0740; Rho-related BTB domain-containing 1	AB018283	Hs.15099	0.25 (0.17–0.37)
Transcription	SNAPC4; small nuclear RNA activating complex, polypeptide 4, 190 kDa	AF032387	Hs.113265	0.32 (0.19–0.54)
	ZNF263; zinc finger protein 263; FPM315	D88827	Hs.182528	0.25 (0.18–0.33)
	IFI35; interferon-induced protein 35	L78833	Hs.50842	0.089 (0.050–0.16)
Unknown	HSPC111; hypothetical protein HSPC111; CGI-117 protein	AI553745	Hs.279918	0.33 (0.13–0.83)
	UGT2B10; UDP glycosyltransferase 2 family, polypeptide B10	X63359	Hs.294039	0.33 (0.15–0.73)
	PP591; hypothetical protein PP591	U79241	Hs.118666	0.32 (0.16–0.62)
	LOC51035: ORF	M68864	Hs.351296	0.26 (0.075–0.92)
	KIAA 0239	D87076	Hs.9729	0.24 (0.061–0.95)
	FLJ10849; hypothetical protein FLJ10849	W25874	Hs.8768	0.23 (0.14–0.37)
	KIAA 0112	D25218	Hs.71827	0.20 (0.099–0.41)
	Retinoic acid-inducible endogenous retroviral DNA	M64936	Hs.367984*	0.19 (0.10–0.35)
	KIAA 0138	D50928	Hs.159384	0.16 (0.093–0.26)

We have included all sequences not tabulated elsewhere that were decreased by at least 0.33-fold and that met our post hoc filtering criteria. *UniGene number for this sequence is from Build 155.

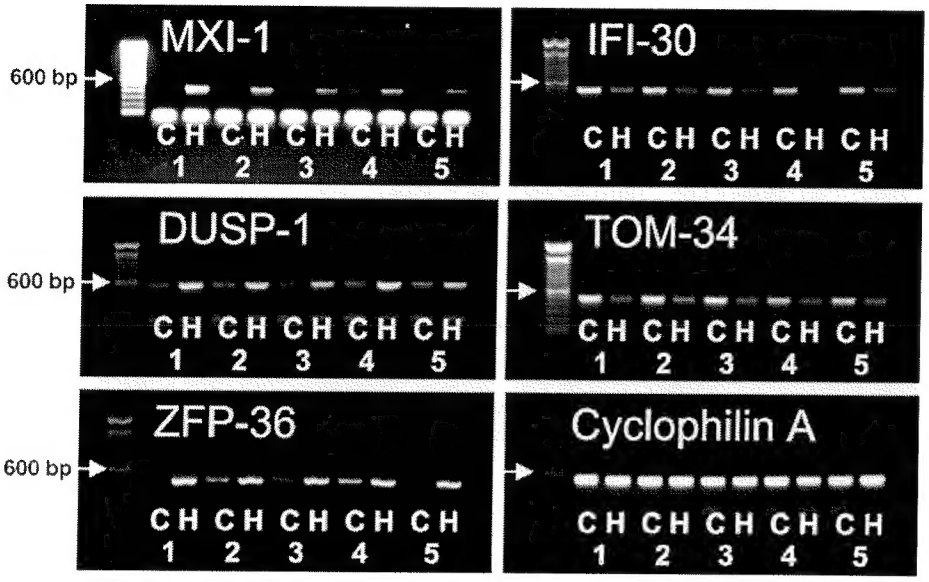

Fig. 2. Confirmatory reverse transcription-PCR. C, control; H, hypoxia. Each paired set of samples is labeled with the experiment number and corresponds to the samples used in Fig. 1. The arrows point to the location of the 600-bp band in the ladder.

cells (34). Twenty-nine sequences (22 decreased, 7 increased) showed a statistically significant change in expression as a result of hypoxic exposure (Table 5). However, four of the seven increased sequences represented the same gene (ubiquitin C). Furthermore, of all the sequences whose expression was significantly altered by hypoxia, only two showed a mean change in expression of twofold or greater: αA-crystallin and APG-1 (Table 5). Of note, glucose-regulated protein (HSPA5) and oxygen-regulated protein 150 (ORP 150) were both highly expressed in the control cells (with mean signal intensities among the top 5% of the sequences present on the GeneChip) and were not increased further by hypoxia.

The lack of a heat shock response to hypoxia was not due to a generalized impairment of the heat shock response in this cell line. As shown in Fig. 3, cells exposed to 43°C for 30 min showed a substantial increase in expression of mRNA HSP-70B′ (HSPA6) that persisted for hours after return to 37°C.

Comparison of the effects of hypoxia to previous reports of heat shock. Mammalian cells are known to respond to environmental stresses such as heat by initiating a program of gene expression commonly referred to as the cell stress response (for recent reviews, see Refs. 4, 13, 33). This response includes both heat shock proteins as well as a number of stress genes not traditionally thought of as HSPs (33). In an effort to judge the extent to which our hypoxic exposure produced a truly nonspecific cell stress response, we compared the genes affected by hypoxia in HepG2 cells to lists of genes which have been identified as part of the cellular response to heat shock in other cell lines (6, 33, 34).

A comparison of the 2,908 sequences that showed a statistically significant change in expression in this study to 2,903 sequences that were previously found to be significantly affected by heat (at 2 h and 40 min after a conventional heat shock) in normal human peripheral blood mononuclear cells (PBMCs) (34), revealed that only 12% (137 increased and 200 decreased) showed changes in expression that were both significant and in the same direction; another 16% (467 sequences) showed opposite changes in expression (significantly increased in one condition, decreased in the other). Likewise, of 37 sequences that were recently reported to be affected by sublethal heat shock in retinal pigment epithelial (RPE) cells (6), we were able to identify comparable sequences on the U95Av2 array for 36, and of these, only 5 (14%) were changed in the same direction by both hypoxia (at 24 h) and by heat shock (during at least one time point in the 24-h recovery period after thermal injury). Seven others (19%) showed opposite changes in expression.

We also compared our results to a published list of non-HSP genes that are affected by heat shock in mammalian systems (33). This list includes genes that exhibit changes in expression both during acute heat exposure as well as during recovery (i.e., after return to normothermic conditions). Of the 58 non-HSP genes on this list, we were able to identify corresponding probe sequences on the GeneChip array for 50, and of these, 11 (22%) showed changes in direction during hypoxia similar to those reported for heat shock. This list included several well-known stress response genes such as DUSP-1, mcl-1, fos, jun, IκBα, VEGF (all increased), and C/EBP-α (decreased). Furthermore, another seven transcripts (14%) showed opposite changes in expression during the two conditions, including ICAM-1 and IL-8 (significantly increased during hypoxia but reported to be decreased by heat shock).

As noted previously, 21 of 26 assessable sequences identified by Semenza (30, 31) as targets of HIF-1 were also found to be upregulated by hypoxia in this experiment. This concordance (81%) was significantly greater than the concordance with non-HSP genes affected by heat shock (22%, $P < 0.001$ by chi-square analysis). This statistically significant difference per-

Table 5. *Effect of hypoxia on HSPs, chaperonins, and cochaperonins*

Functional Class	Common Name(s)	GenBank or TIGR No.	UniGene No.	Change in Expression, fold (geometric mean, 95% CI)
HSP 10	HSPE1; heat shock 10-kDa protein 1; chaperonin 10; HSP 10	AI912041	Hs.1197	0.72 (0.60–0.87)*
HSP 20 family	CRYAA; crystallin, alpha A	U05569	Hs.184085	2.1 (1.2–3.6)*
	CRYAB; crystallin, alpha B	AL038340	Hs.1940	0.68 (0.31–1.5)
	CRYAB; crystallin, alpha B	AL038340	Hs.1940	1.1 (0.90–1.4)
	HSPB1; heat shock 27-kDa protein 1	Z23090	Hs.76067	0.97 (0.68–1.4)
	HSPB2; heat shock 27-kDa protein 2	S67070	Hs.78846	1.1 (0.77–1.6)
	HSPB2; heat shock 27-kDa protein 2	S67070	Hs.78846	1.2 (1.0–1.5)*
	HSPB3; heat shock 27-kDa protein 3	U15590	Hs.41707	1.0 (0.51–2.1)
	HSPB3; heat shock 27-kDa protein 3	Y17782	Hs.41707	0.74 (0.34–1.6)
HSP 32	Heme oxygenase-1	Z82244	Hs.202833	1.2 (0.40–3.6)
HSP 40 family	DNAJA1; DnaJ (Hsp40) homolog, subfamily A, member 1	L08069	Hs.94	0.56 (0.42–0.74)*
	DNAJA1; DnaJ (Hsp40) homolog, subfamily A, member 1	L08069	Hs.94	0.52 (0.38–0.72)*
	DNAJB1; DnaJ (Hsp40) homolog, subfamily B, member 1	D85429	Hs.82646	0.85 (0.47–1.5)
	DNAJB4; DnaJ (Hsp40) homolog, subfamily B, member 4	U40992	Hs.41693	1.2 (0.69–1.9)
	DNAJB4; DnaJ (Hsp40) homolog, subfamily B, member 4	U40992	Hs.41693	0.93 (0.22–3.9)
	DNAJB5; DnaJ (Hsp40) homolog, subfamily B, member 5	AF088982	Hs.237506	1.3 (0.51–3.2)
	DNAJB6; DnaJ (Hsp40) homolog, subfamily B, member 6; MRJ	AI540318	Hs.181195	0.93 (0.64–1.4)
	DNAJB6; DnaJ (Hsp40) homolog, subfamily B, member 6; MRJ	AB014888	Hs.181195	0.84 (0.72–0.97)*
	DNAJB9; DnaJ (Hsp40) homolog, subfamily B, member 9	AL080081	Hs.6790	0.97 (0.60–1.6)
	DNAJC9; DnaJ (Hsp40) homolog, subfamily C, member 9	AI680675	Hs.44131	0.42 (0.32–0.56)*
HSP 47	HSP 47; colligin 1; SERPINH1	X61598	Hs.241579	0.84 (0.39–1.8)
	HSP 47; colligin 2; SERPINH2	D83174	Hs.9930	1.1 (0.95–1.2)
	HSP 47; colligin 2; SERPINH2	D83174	Hs.9930	1.6 (1.4–2.0)*
HSP 56	HSP 56; immunophilin; FKBP52; FKBP4	M88279	Hs.848	0.84 (0.68–1.0)
HSP 60 family	HSPD1; HSP 60-1; Mitochondrial matrix protein P1	M22382	Hs.79037	0.96 (0.81–1.1)
	CCTB; chaperonin-containing TCP-1 subunit 2; CCT2	AF026166	Hs.6456	0.82 (0.71–0.96)*
	CCTD; chaperonin-containing TCP-1 subunit 4; CCT4	AF026291	Hs.79150	0.82 (0.71–0.96)*
	CCTE; chaperonin-containing TCP-1 subunit 5; CCT5	D43950	Hs.1600	0.69 (0.58–0.82)*
	CCTG; chaperonin-containing TCP-1 subunit 3; CCT3	X74801	Hs.1708	0.72 (0.64–0.80)*
	T-complex polypeptide 1; TCP-1	X52882	Hs.4112	0.63 (0.55–0.72)*
	TCP-20; CCT6A	L27706	Hs.82916	0.61 (0.51–0.74)*
HSP 70 family	HSPA1A; heat shock 70-kDa protein 1A	M11717	Hs.8997	0.99 (0.18–5.5)
	HSPA1B; heat shock 70-kDa protein 1B	M59830	Hs.274402	1.0 (0.20–5.1)
	HSPA1B; heat shock 70-kDa protein 1B	W28645	Hs.274402	0.63 (0.10–3.9)
	HSPA1L; HSP 70 homolog	D85730	Hs.80288	0.90 (0.57–1.4)
	HSPA2; heat shock 70-kDa protein 2	L26336	Hs.75452	0.79 (0.37–1.7)
	HSPA2; heat shock 70-kDa protein 2	L26336	Hs.75452	0.47 (0.15–1.5)
	HSPA4; heat shock 70-kDa protein 4	L12723	Hs.90093	0.71 (0.55–0.91)*
	HSPA5; glucose regulated protein, 78kD	X87949	Hs.75410	1.1 (0.79–1.6)
	HSPA6; heat shock 70-kDa protein 6; HSP 70-B′	X51757	Hs.3268	1.6 (0.72–3.6)
	HSPA6; heat shock 70-kDa protein 6; HSP 70-B′	X51757	Hs.3268	1.6 (0.47–5.5)
	HSPA8; heat shock 70-kDa protein 8; HSC 70	HT2995	Hs.180414	0.60 (0.38–0.94)*
	HSPA8; heat shock 70-kDa protein 8; HSC 70	HT2995	Hs.180414	0.57 (0.35–0.93)*
	HSPA8; heat shock 70-kDa protein 8; HSC 70	W28493	Hs.180414	0.78 (0.13–4.5)
	HSPA8; heat shock 70-kDa protein 8; HSC 70	Y00371	Hs.180414	0.60 (0.40–0.90)*
	HSPA9B; heat shock 70-kDa protein 9B; mortalin 2	L15189	Hs.3069	0.77 (0.62–0.95)*
	ORP150; oxygen regulated protein (150kD)	U65785	Hs.277704	0.95 (0.78–1.1)
HSP 90 family	HSPCA; heat shock 90-kDa protein 1, alpha; HSP 90-1-alpha	X15183	Hs.289088	0.71 (0.61–0.82)*
	HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta	M16660	Hs.74335	1.0 (0.89–1.2)
	HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta	J04988	Hs.74335	1.1 (0.88–1.3)
	HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta	W28616	Hs.74335	1.1 (0.69–1.9)
	HSPCB; heat shock 90-kDa protein 1, beta; HSP 90-1-beta	W28616	Hs.74335	1.0 (0.82–1.3)
HSP 105	HSP105B; heat shock 105-kDa, HSP 105; KIAA 0201	D86956	Hs.36927	0.39 (0.11–1.4)
HSP 110 family	APG-1; heat shock protein (hsp110 family); HSP 110	AB023421	Hs.71992	0.46 (0.28–0.74)*
	APG-2; heat shock 70-kDa protein 4; HSPA4	AB023420	Hs.90093	0.64 (0.15–2.7)
Ubiquitins	UBB; ubiquitin B	U49869	Hs.356190	0.81 (0.72–0.91)*
	UBB; ubiquitin B	X04803	Hs.356190	0.81 (0.74–0.89)*
	UBC; ubiquitin C	M26880	Hs.183704	1.4 (1.0–1.9)*
	UBC; ubiquitin C	M26880	Hs.183704	1.3 (1.1–1.6)*
	UBC; ubiquitin C	AB009010	Hs.183704	1.3 (1.0–1.6)*
	UBC; ubiquitin C	AB009010	Hs.183704	1.3 (1.1–1.4)*
HSP binding protein	HSPBP1; HSP 70-interacting protein	AF093420	Hs.53066	0.99 (0.61–1.6)
Other	Alpha-2 macroglobulin receptor associated protein-1	M63959	Hs.75140	0.98 (0.89–1.1)
	TBCC; tubulin-specific cofactor (chaperone) C	U61234	Hs.75064	0.85 (0.65–1.1)
	TBCD; tubulin-specific cofactor (chaperone) D	AB023205	Hs.12570	0.79 (0.65–0.96)*
	PPIA; peptidylprolyl isomerase A; cyclophilin A	X52851	Hs.342389	0.98 (0.93–1.0)
	P23; unactive progesterone receptor, 23 kDa	L24804	Hs.278270	0.93 (0.67–1.3)
	ST-13; SNC-6; HSP70-interacting protein; P48	U17714	Hs.119222	0.98 (0.93–1.0)
	HSP70/HSP90 organizing protein; stress-induced phosphoprotein 1	M86752	Hs.355930	0.64 (0.49–0.85)*

*Statistically significant change in expression

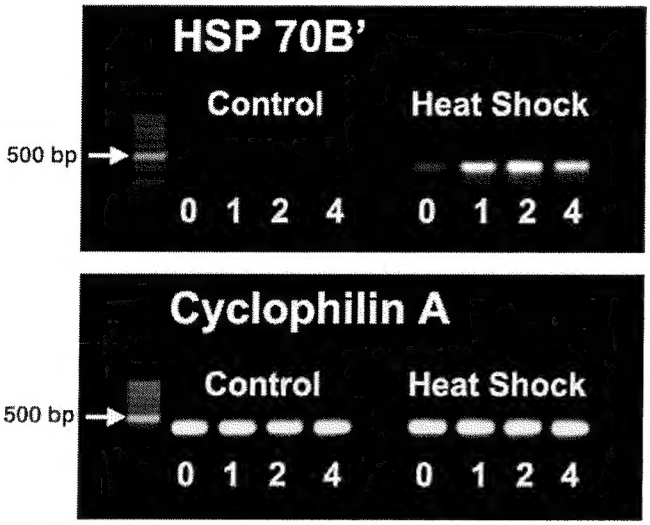

Fig. 3. RT-PCR showing the effect of heat shock on expression of HSP 70B′ by HepG2 cells. Cells were exposed to control conditions (37°C) or heat shock (43°C) for 30 min and then returned to control conditions. The numbers refer to the time after return to 37°C at which the sample was taken (in hours). The arrows point to the 500-bp band in the ladder. Similar results were obtained in two other, separate experiments.

sisted even when we included the list of downregulated genes reported by Fink et at. (9) in our analysis ($P < 0.001$ by chi-square).

DISCUSSION

The results of this study suggest that the cellular response to hypoxia at the level of transcript expression is even broader than previously realized, yet may also be more specific to hypoxia than generally appreciated. Furthermore, as noted by others (e.g., Ref. 9) this response includes a substantial number of transcripts with decreased expression.

One of the greatest risks of an experiment that simultaneously assesses the expression of thousands of different sequences is the possibility of false-positive results. By random chance alone, one might expect 5% of sequences (about 630, in this case) to appear to be affected by any given stimulus. To address this issue, we subjected our findings to both external validation (comparison to lists of genes previously shown to be affected by hypoxia as well as to genes not expected to be affected by hypoxia) and to internal validation (replication of select findings by RT-PCR) and applied post hoc filters that we believe will minimize the number of false-positive reports in Tables 3 and 4. Indeed, the 95% confidence intervals reported in these Tables are generally well removed from onefold. Furthermore, the fact that our unfiltered analysis appropriately detected changes in expression where expected (Table 2) but not generally in housekeeping sequences (Table 1), coupled with the observation that a number of the sequences identified after post hoc filtering (in Table 3) have been found to be affected by hypoxia in other systems, increases our confidence that the novel findings reported here are likely to be true-positive results. This analytic approach has previously yielded highly satisfactory results in normal human cells subjected to heat shock (34).

Two important limiting issues must be kept in mind when interpreting these data. We studied an immortal tumor cell line which, though well characterized and conventionally studied, may in some ways respond differently to hypoxia than a terminally differentiated cell line. Furthermore, our control conditions (room air-5% CO_2 incubator), although experimentally conventional, subject cells to a Po_2 that is higher than the 12–30 mmHg (the equivalent of ~2–4% Fio_2) typically reported in the interstitial fluid of normoxic organs (30). It is therefore possible that some of the apparent downregulation of gene expression by hypoxia in fact represents the effects of hyperoxic upregulation of genes in the control cells.

Although differing in some of the particulars, our data confirm and extend the findings of Fink et al. (9) in two very important respects. First, we found that hypoxia has a substantial downregulatory effect on transcript expression in HepG2 cells in addition to its well-known upregulatory effects. Prior to post hoc filtering, the number of transcripts that showed a statistically significant decrease in expression was greater than the number of transcripts that showed a statistically significant increase in expression. Furthermore, there was a statistical trend ($P = 0.12$) toward a decrease in the total number of transcripts expressed by the cells exposed to hypoxic conditions. In principle, such a downregulatory effect could be produced by decreasing expression uniformly across the entire population of cells or by causing a marked decrease in gene expression in a subset of cells within the population, as would occur if some of the cells were induced to undergo apoptosis. Although we were unable to distinguish between these two possibilities using trypan blue exclusion, it is noteworthy that Bae et al. (3) have found evidence that exposure of HepG2 cells to 1% hypoxia for 24 h induces apoptotic changes in about 30–40% of cells in culture. It is therefore possible that some of the downregulatory effect of hypoxia that we and others have noted in this cell line is due to induction of apoptosis in a subset of the cells in culture.

The second important area of concordance between our results and those of Fink et al. (9) was the lack of a generalized induction of HSPs by exposure to 1% oxygen for 24 h. On the contrary, almost half of the HSPs and chaperonins examined that were expressed under control conditions showed a decrease in expression under hypoxic conditions. We would not have missed a generalized increase in HSP expression had one been present, as HepG2 cells are capable of producing a vigorous increase in HSP 70B′ expression in response to a conventional heat shock (Fig. 3), and we have previously detected changes in HSP expression in PBMCs using an almost identical (U95A) Affymetrix array (34). However, it is also possible that the hypoxic stimulus, although conventional, was insufficiently severe to induce a heat

shock response in this cell line or that the time point we chose to examine was inadequate to detect a heat shock response. In this regard, it is noteworthy that Patel et al. (25) have reported induction of HSP-70 RNA in HepG2 cells under conditions of anoxia (0% O_2), with expression peaking at 6 h.

A comparison of our results to the heat shock literature supports the hypothesis that the human cellular response to stress involves both changes in expression that are stress specific (and perhaps even cell type specific) as well as changes that are truly nonspecific to the cell type and stressor applied. The existence of stress-specific changes in expression is not surprising, as the most important transcription factors activated by hypoxia and heat [HIF-1 (30, 31) and HSF-1 (20, 26), respectively] are distinct from each other. However, our findings also suggest that the truly nonspecific component of the human cell stress response is possibly quite small, amounting to somewhere between 10 and 15% of the total number of genes affected by a given environmental stressor. We believe that identifying and characterizing these nonspecific genes is important, as doing so may provide novel insights into how cells acquire cross-tolerance to multiple environmental stressors. Conversely, genes that are similarly affected by multiple different stressors are potentially poor targets for therapeutic manipulations that are aimed at adaptations to a particular stress [such as cytotoxic therapy directed at hypoxically adapted cells, as proposed by Wouters et al. (39)]. Experiments that are specifically designed to catalog and distinguish stress-specific and stress-nonspecific gene expression responses may be of considerable interest and potentially of practical value, particularly under conditions of compensable stress such as those studied in this experiment.

In conclusion, we have found that the response of human HepG2 cells to hypoxia at the level of mRNA expression is extensive and includes a significant component of downregulation. Interestingly, hypoxia did not induce a generalized heat shock response in HepG2 cells compared with control conditions, and the degree of overlap between the responses of HepG2 cells to 24 h of exposure to 1% oxygen and the known responses of mammalian cells to heat shock appears to be small. Our findings are consistent with the concept that the response of human cells to environmental stress includes both stress-specific and nonspecific components. In human cells, this nonspecific component may in fact be much smaller than generally recognized.

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